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WITNESS my hand this
Thirtieth day of November 1999

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The Walter and Eliza Hall Institute of Medical Research

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"A method of activating T cells and agents useful for same"

The invention is described in the following statement:

A METHOD OF ACTIVATING T CELLS AND AGENTS USEFUL FOR SAME

The present invention relates generally to a method of activating T cells and more particularly to a method of activating T cells using glycosylphosphatidylinositol (referred to herein as "GPI") molecules and derivatives thereof. Even more particularly the method of the present invention contemplates a method activating T cells, using GPI molecules, via a CD1-restricted pathway. The method of the present invention is useful, *inter alia*, in the development of vaccines for therapeutic and prophylactic applications.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Antibody responses to protein are understood to be MHC restricted. That is, the production of antibodies directed to a given T dependent antigen requires the production of cytokines by stimulated TH2 cells. Said TH2 cells are stimulated following their binding to a MHC II/peptide complex comprising a peptide derived from the processing of said antigen. Since MHC molecules are polymorphic, there exist genetically determined high and low responders to peptide vaccines.

GPI anchor surface proteins occur frequently among medically important parasitic and fungal taxa such as *Plasmodium, Trypanosoma, Leishmania, Toxoplasma* and *Candida.*

GPIs are ubiquitous among eukaryotes, described from *T. brucei, T. cruzi, Plasmodium, Leishmania*, and *Toxoplasma*, as well as yeast, insect, fish and numerous mammalian sources (for recent reviews see (1, 2)). GPIs consist of a conserved core glycan (Manα1-2Manα1-6Manα1-4GlcNH_2 linked to the 6-position of the *myo*-inositol ring of PI. GPIs

are built up on the cytoplasmic face of the endoplamic reticulum by the sequential addition of sugar residues to PI by the action of glycosyltransferases. The maturing GPI is then translocated across the membrane to the luminal side of the ER, whence it may be exported to the cell surface, free or in covalent association with proteins. The terasaccharide core glycan may be further substituted with sugars, phosphates and ethanolamine groups in a species and tissue-specific manner. GPI fatty acid moieties can be either diacylglycerols, alkylacylglycerols, monoalkylglycerols or ceramides, with additional palmitoylations or myristoylations to the inositol ring. The overall picture is of a closely related family of glycolipids sharing certain core features but with a high level of variation in fatty acid composition and side-chain modifications to the conserved core glycan.

In work leading up to the present invention, the inventors have shown that the antibody response to several parasitic proteins is regulated predominantly through CD1-restricted recognition of the covalently associated GPI moiety by IL-4 producing CD4+ T cells with limited TCR repertoire diversity. In contrast, there is little evidence for MHC II restricted T cell responses to these antigens. GPI moieties, therefore, act as universal T cell sites through presentation by the non-polymorphic CD1 restriction element.

Accordingly, one aspect of the present invention contemplates a method of activating T cells comprising administering a T cell activating effective amount of a molecule or a complex comprising said molecule which molecule or molecule complex is capable of interacting with CD1 on an immune cell to form an association with CD1 which association activates helper T cells.

Preferably, the present invention contemplates a method of activating T cells comprising administering a T cell activating effective amount of GPI or a complex comprising GPI which GPI or GPI-complex is capable of interacting with CD1 on an immune cell to form an association with CD1 which association activates helper T cells.

Reference hereinafter to "activating" helper T cells is a reference to upregulating the functions which said T cells are capable of performing upon stimulation by an antigen, such as, but not

limited to, one or more of cell division, differentiation, cell surface molecule expression or cytokine production.

Reference to "association" should be understood in its broadest sense to include any form of interaction between a molecule and CD1. Said molecule and CD1 may interact via, for example, a covalent bond, ionic bond, hydrogen bond, van Der Waals forces or other interactive bonding mechanism.

Reference hereinafter to "GPI" should be read as including reference to all forms of GPI and derivatives thereof. Derivatives include fragments, parts, portions, chemical equivalents, mutants, homologs and analogs. Chemical equivalents of GPI can act as a functional analog of GPI. Chemical equivalents may not necessarily be derived from GPI but may share certain conformational similarities. Alternatively chemical equivalents may be specifically designed to mimic certain physiochemical properties of GPI. Chemical equivalents may be chemically synthesised or may be detected following, for example, natural product screening. Homologs of GPI contemplated herein include, but are not limited to, GPI from different species.

GPI molecules suitable for use in the present invention may be derived from any natural or synthetic source. This includes, for example, GPI moieties derived by genetic manipulation of expression systems and by manipulations of the GPI post-translational modification of proteins via recombinant DNA techniques such as glycosylation inhibitors. Examples of GPI moieties suitable for use in the present invention include but are not limited to microorganism GPI moieties which cause disease conditions such as, the parasitic, fungal and yeast taxa *Plasmodium*, *Trypanosoma*, *Leishmania*, *Toxoplasma* and *Candida*. Preferably, said GPI is a parasite GPI and even more preferably a *Plasmodium* GPI.

According to this preferred embodiment, the present invention contemplates a method of activating T cells comprising administering a T cell activating effective amount of *Plasmodium* GPI or a complex comprising said *Plasmodium* GPI which *Plasmodium* GPI

or *Plasmodium* GPI complex is capable of interating with CD1 on an immune cell to form an association with CD1 which association activates helper T cells.

Most preferably said *Plasmodium* is *P. falciparum*.

Said GPI "complex" is a reference to a GPI moiety coupled to any other molecule. Said molecule may be any molecule to which an immune response is sought, for example, a carbohydrate or a peptide, polypeptide or protein such as, but not limited to, peptides, polypeptides or proteins naturally anchored to GPI moieties (for example, malarial CS protein, MSP-1, MSP-2, *Leishmanial* PSA-2 or GP63) or any peptides, polypeptides or proteins artificially coupled to a GPI moiety (for example an influenza antigen). Said molecule and said GPI moiety may be covalently linked or may be linked by ionic, hydrogen or other interactive bonding mechanisms. Coupling may be achieved by a variety of techniques including, but in no way limited to, use of a specific expression system or via chemical synthesis. Preferably, said molecule is a protein.

Reference to an "immune cell" should be understood as a reference to any cell of the immune system such as, but not limited to, myeloid cells, stromal cells or antigen presenting cells (for example macrophages).

Reference to "helper T cells" should be understood as a reference to any cell expressing a T cell receptor (expression of a "T cell receptor" is defined as the expression of one or more of an α, β, γ and/or δ T cell receptor chain in either homodimeric or heterodimeric form) which can become activated via a CD1-restricted recognition pathway instead of, or in addition to, a capacity to become activated via a MHC II restricted recognition pathway and which acts to stimulate, upregulate or otherwise modulate any aspect of the immune response via any one or more of a variety of mechanisms including, for example, cell-cell contact or production of soluble mediators. Said T cells include, but are not limited to, thymically derived T cells. Preferably said T cells express CD4 and even more preferably CD4 and NK1.1.

According to this most preferred embodiment, the present invention contemplates a method of activating T cells comprising administering a T cell activating effective amount of GPI or a complex comprising said GPI which GPI or GPI complex is capable of interacting with CD1 on an immune cell to form an association with CD1 which association activates CD4+ NK1.1+ T cells.

Without limiting the present invention to any one theory or mode of action, administration of GPI or a GPI complex leads to presentation of the GPI moiety, or a molecule complexed to the GPI moiety, by a non-polymorphic CD1 restriction element. CD1 is expressed on a variety of cells including, for example, macrophages. Coupling of the GPI moiety (or the molecule complexed to the GPI) to the CD1 may be by covalent bonding. Recognition of the CD1-GPI unit by a subclass of CD4+ T cells leads to their activation. Said T cells do not recognise the GPI moiety or the molecule complexed to the GPI via the traditional MHC II restricted route of presentation, instead said T cells are activated through CD1-restricted recognition. Said T cells represent a subset of the T cell population of an individual. For example, T cells exhibiting the phenotype CD4+, NK1.1+ are able to become activated via the CD1-restricted recognition pathway. The present invention should be understood to extend to methods of activating T cells by administration of GPI or GPI complex wherein said T cells are activated by one or both of CD1 or MHC II restricted recognition.

Even more preferably the present invention contemplates a method of activating CD4+, NK1.1+ T cells comprising administering a T cell activating effective amount of a *Plasmodium* GPI or a complex comprising said *Plasmodium* GPI which *Plasmodium* GPI or *Plasmodium* GPI complex is capable of interacting with CD1 on an immune cell to form an association with CD1 which association activates CD4+ NK1.1+ T cells.

Most preferably said *Plasmodium* is *P. falciparum*.

Without limiting the present invention to any one theory or mode of action, the T cells of

the present invention can co-operate with B cells to result in CD1-restricted antibody production, the specificity of said antibody being directed to the GPI moiety, where said GPI is administered in isolation, or to a molecule complexed to the GPI moiety where a GPI complex is administered. For example, said complexed molecule may be a protein to which an antibody response is desired, such as an influenza antigen. Activation of said B cells is supported by the production of cytokines, such as IL-4, by the CD1-restricted activated T cells. GPI-anchoring, therefore, permits antibody formation by a non-MHC restricted immunological process. Unlike MHC, which is a highly polymorphic molecule, CD1 is non-polymorphic thereby resulting in little observed non-responsiveness in human populations.

Accordingly, a related aspect of the present invention contemplates a method of activating CD4+, NK1.1+ T cells comprising administering a T cell activating effective amount of a GPI or a complex comprising said GPI which GPI or GPI complex is capable if interacting with CD1 on an immune cell to form an association with CD1 which association activates CD4+, NK1.1+ T cells wherein said activated T cells provide B cell help.

Preferably, said GPI molecule is a parasitic, fungal or yeast GPI moiety. Most preferably, said GPI molecule is a parasite GPI and even more preferably a *plasmodium* GPI.

Accordingly, a related aspect of the present invention contemplates a method of activating CD4+, NK1.1+ T cells comprising administering a T cell activating effective amount of a *Plasmodium* GPI or a complex comprising said *Plasmodium* GPI which *Plasmodium* GPI or *Plasmodium* GPI complex is capable of interacting with CD1 on an immune cell to form an association with CD1 which association activates CD4+, NK1.1+ T cells wherein said activated T cells provide B cell help.

Most preferably said *Plasmodium* is *P. falciparum*.

Reference to "B cell help" should be understood as a reference to the provision, by said activated T cells, of signals which act to stimulate, up-regulate or otherwise modulate or maintain B cell and/or plasma cell viability or functional activity. Said signals may take any form including, for example, cell/cell contact or the production of soluble mediators such as cytokines.

A further aspect of the present invention relates to the use of the invention in relation to disease conditions. For example:

(i) As vaccines to molecules naturally GPI-anchored such as, for example, the malarial CS protein, MSP-1, and MSP-2, *Leishmanial* PSA-2 and gp63.

(ii) As vaccines to GPI molecules themselves, for example in treating parasitic, fungal and yeast infections.

(iii) To enable antibody production where MHC II restricted T cell activation is undesirable, such as where said MHC II restricted T cell would engender an autoimmune reaction. For example, the M protein of Group A Streptococcus exhibits T cell epitopes which are cross-reactive with human heart tissue but exhibits B cell epitopes protective against the bacterium. Said B cell epitope may be coupled to GPI to facilitate non-MHC restricted antibody formation.

(iv) To provide a universal T cell epitope for any vaccine construct.

Accordingly, another aspect of the present invention contemplates a method of treating a mammal said method comprising administering to said mammal an effective amount of GPI or a complex comprising said GPI which GPI or GPI complex is capable of interacting with CD1 on an immune cell to form an association with CD1 which association activates helper T cells.

Preferably said mammal has a parasitic infection and more preferably a *Plasmodium* infection.

Most preferably said GPI is *Plasmodium* GPI.

Accordingly, the present invention contemplates a method of treating a mammal having a *Plasmodium* infection said method comprising administering to said mammal an effective amount of *Plasmodium* GPI or a complex comprising *Plasmodium* GPI which *Plasmodium* GPI or *Plasmodium* GPI complex is capable of interacting with CD1 on an immune cell to form an association with CD1 which association activates helper T cells.

Another aspect of the present invention contemplates a method of treating a mammal said method comprising administering to said mammal an effective amount of *Plasmodium* GPI or a complex comprising *Plasmodium* GPI which *Plasmodium* GPI or *Plasmodium* GPI complex is capable of interacting with CD1 on an immune cell to form an association with CD1 which association activates helper T cells wherein said activated T cells provide B cell help.

In another aspect the present invention contemplates a method of treating a mammal said method comprising administering a mammalian helper T cell activation effective amount of a GPI or a complex comprising said GPI which GPI or GPI complex is capable of interacting with CD1 on an immune cell to form an association with CD1 which association activates helper T cells.

Preferably said mammal has a parasitic infection and more preferably a *Plasmodium* infection.

Most preferably said GPI is *Plasmodium* GPI.

According to this most preferred embodiment the present invention contemplates a method of treating a mammal having a *Plasmodium* infection said method comprising administering a mammalian helper T cell activation effective amount of a *Plasmodium* GPI or a complex comprising said *Plasmodium* GPI which *Plasmodium* GPI or *Plasmodium* GPI complex is capable of interacting with CD1 on an immune cell to form an association with CD1 which association activates helper T cells.

Yet another aspect of the present invention contemplates the use of GPI or a complex comprising said GPI which GPI or GPI complex is capable of interacting with CD1 on an immune cell, in the manufacture of a medicament for the activation of helper T cells in a mammal.

Preferably said mammal has a parasitic infection and more preferably a *Plasmodium* infection.

Most preferably said GPI is *Plasmodium* GPI.

The term "mammal" includes humans, primates, livestock animals (e.g. horses, cattle, sheep, pigs and donkeys) laboratory test animals (e.g. mice, rats, rabbits, guinea pigs) companion animals (e.g. dogs and cats) and captive wild animals (e.g. kangaroos, deer, foxes). Preferably, the mammal is a human or laboratory test animal. Even more preferably the mammal is a human.

In a related aspect of the present invention the mammal undergoing treatment may be human or an animal in need of therapeutic or prophylactic treatment.

Accordingly, another aspect of the present invention relates to a vaccine composition comprising as the active component GPI or GPI complex, as broadly described above, together with one or more pharmaceutically acceptable carriers and diluents.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be

incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells. The vector may, for example, be a viral vector.

The present invention is further described by the following non-limiting Examples:

EXAMPLE 1

REAGENTS AND ANIMALS

Pronase was obtained from Boehringer Mannheim. Octyl-Sepharose, Protein-G Sepharose, *n*-octylthiogluco-pyranoside (*n*-otg), phenylmethylsulfonylfluoride (PMSF), *p*-tosyl-L-lysinechloromethylketone (TLCK), N-tosyl-L-phenylalaninechloro-methylketone (TPCK), *p*-chloromercuriphenylsulphonic acid (*p*-CMPS), aprotinin, leupeptin, pepstatin, iodoacetamide, iodoacetic acid and *n*-ethylmaleimide (NEM) were obtained from Sigma Chemical Co. Sephadex was from Pharmacia. Analytical or HPLC grade, acetic acid, butanol, chloroform, diethyl ether, ethanol, methanol and water were obtained from BDH and Waters. Silica G60 TLC plates were from Merck Darmstadt. Tritiated mannose, glucosamine, myristic and palmitic acids were from Amersham. The recombinant *P. falciparum* CS protein 2.3 consists of the entire gene except for the C-terminal 21 amino acids. The *P. berghei* rCS encompasses amino acids 81-277, including the central repetitive domain. The $NANP_{40}$ peptide, and the 17-mer peptide of the tandemly repeating domain of the *P. berghei* CS protein $(DPPPPNPN)_2D$, were synthesized by routine methods.

Adult female C57Bl/6 wild-type mice, C57Bl/6 lacking the MHC Class II gene, or the CD1.1 and CD1.2 genes, congenic mice on the C57Bl/10 or Balb background, Balb/c *nu/nu* mice and other mouse haplotypes used in the study were maintained in specific pathogen free animal facilities.

EXAMPLE 2

CHIMERAS

Bone marrow was derived from the femurs and tibiae of Balb/c, Balb/B, Balb/K donors. T cells and NK cells were depleted by complement-mediated lysis of Thy-1^+, $CD4^+$, Lyt.2^+ and asialo-GM^+ cells. Bone marrow aspirates in RPMI 1640 + 3% BSA at 10^7 cells/ml were incubated on ice with the appropriate dilution of specific antibody, followed

by centrifugation at 2°C and resuspension in low toxicity, mouse lymphocyte absorbed, sterile-filtered Guinea pig complement at 37°C in RPMI 1640 + 3% BSA for 1 hr. Syngeneic or allogeneic recipients, maintained on acidified water and tetracycline, were irradiated by cobalt source (1000 Rads) and inoculated i.v. with 10^7 T-depleted cells. Animals were maintained on acidified water and rested for 12 weeks prior to testing for chimerism and use in experiments. To generate thymic chimeras, thymic lobes were obtained by dissection from neonatal Balb/c, Balb/B, and Balb/K mice and implanted into the interscapular dorsum of adult Balb/c *nu/nu* mice via a subcutaneous incision. Mice were maintained in sterile isolators on acidified water and rested 12 weeks prior to testing for chimerism and use in experiments.

EXAMPLE 3

PREPARATION OF BLOOD-STAGE *P. FALCIPARUM* PARASITES

The FCB-1 line of *Plasmodium falciparum* were grown *in vitro* by the method of Trager and Jensen. Synchronous development of parasites was maintained by the sorbitol method of Lambros and Vanderberg. For the biosynthetic labelling of parasite proteins, ^{3}H-palmitic acid conjugated to defatted bovine serum albumin in molar ratio 1:1, ^{3}H-glucosamine or ^{3}H-mannose were added at a final specific activity of 10μCurie/ml, to RPMI 1640 cultures of $2x10^{10}$ parasites at the late trophozoite/early schizont stage for 2 hours (for labelling of GPI precursors) or 8 hours (for labelling of protein-bound GPI). Parasites were harvested by 0.05% Saponin lysis and centrifugation in the cold at 15,000g for 20 minutes, followed by two washes in PBS and storage at -70°C.

EXAMPLE 4

PURIFICATION OF THE 195KD MSP-1 AND 56KD MSP-2 ANTIGENS

The GPI-anchored MSP-1 and MSP-2 merozoite surface proteins were purified to homogeneity. Biosynthetically labelled malaria parasites at the late schizont stage were lysed in 0.05% Saponin and centrifuged at 15,000g for 20 minutes, and washed as above.

The pellet was extracted in 25mM *n*-octyl-thioglucopyranoside (*n*-otg), 1% BSA, 1mM EDTA, 0.1mM EGTA, 1mM PMSF, 1mM TPCK, 0.1mM TLCK, 5mM pCMPS, 1μg/ml pepstatin, 1μg/ml leupeptin, 1mM NEM, 5mM iodoacetamide, 150mM NaCl, 25mM Tris/HCl pH 7.4 by sonication on ice. The extact was clarified by centrifugation at 20,000g for 30 minutes in the cold, and the supernatant decanted and loaded onto two immunoaffinity columns arranged in sequence, containing approximately 10mg monoclonal antibody 111.4 or monoclonal antibody 113.1, each cross-linked to Protein G-Sepharose by gluteraldehyde (all procedures on ice). The protein extract was passed through the column at a rate of 0.3ml/min. The columns were washed first with with 100ml 10mM n-otg, 1% BSA, 300mM NaCl, followed by 100ml 10mM n-otg, 300mM NaCl. Antigen was eluted from each column with four column volumes of 10mM n-otg, 200mM glycine pH 2.8. The pH of the eluate was neutralized with 2M Tris. Aliquots of protein were analysed for purity by SDS-PAGE followed by staining with Coomassie brilliant blue. The remaining purified proteins were dialysed exhaustively against 100mM NH_4HCO_3 using dialysis membrane previously boiled exhaustively in 10mM EDTA followed by boiling in 10 changes of double distilled water. Protein concentration was determined by the method of Bradford.

EXAMPLE 5

PURIFICATION OF MEMBRANE-FORM VARIANT SURFACE GLYCOPROTEIN (MF VSG) OF T. BRUCEI

T. brucei 118 (MITAT 1.5) was purified from the blood of infected Wistar rats by DEAE chromatography. $1x10^{11}$ parasites were pre-incubated for 30 minutes in glucose-deficient RPMI 1640 supplemented with 40mM fructose and then labelled in the same medium for 90 minutes either with [^{3}H]-myristic acid conjugated to defatted BSA, or with [^{3}H]-glucosamine. In the latter case the medium contained 1μg/ml tunicamycin. Parasites were washed in cold medium without label, and taken up in 10mM $ZnCl_2$, followed by centrifugation at 45,000g. The pellet was put into boiling 25mM n-otg, 5mM p-CMPS, 1mM PMSF, 1mM TLCK, 50mM Tris/HCL and allowed to cool, and centrifuged at

45,000g at 2°C. The remaining detergent soluble extract was made up to 1mM $CaCl_2$, 1mM $MgCl_2$, and 1mM $MnCl_2$, and passed over a Con-A sepharose column, followed by washing with 10 column volumes of extraction buffer. The column was first eluted with detergent buffer containing 0.5M α-methyl-mannopyranoside and 0.5M α-glucopyranoside, followed by 25mM *n*-otg in 8M urea. Aliquots were subject to SDS-PAGE and fluorography or staining with Coomassie blue.

EXAMPLE 6

PURIFICATION OF THE C-TERMINAL GPI ANCHORS OF DEFINED PARASITE ANTIGENS

GPI-anchored *P. falciparum* MSP-1, MSP-2 and *T. brucei* 118 (MITat 1.5) mfVSG were labelled with fatty acid or glucosamine as required and purified as above, to 10mg/ml. 600µl methanol was added to 150µl aliquots followed by 150µl $CHCl_3$ and 450µl H_2O. The samples were vortexed and microfuged, the supernatant taken for scintillation counting, and the interphase and lower phase mixed with 450µl methanol and re-centrifuged. The pellet was repeatedly extracted with CMW 10:10:3 until partitioning of fatty-acid label into the supernatant was minimal, partitioned between water and water-saturated butanol, precipitated with acetone at -20°C, and the proteins taken up by sonication in 6M Urea, 1mM DTT, 1mM iodoacetic acid. After 15 minutes at room temperature, the sample was diluted 6 fold and made to 5mM $CaCl_2$. 2.5% pre-digested Pronase B was added, and incubated for 72h at 37°C with 2 additions of 0.25% pronase. The sample was loaded in 5% 1-propanol, 0.1M NH_4OAc onto pre-equilibrated Octyl-Sepharose, washed and eluted in a linear gradient of 1-propanol (5-60%) in water. GPIs eluted at 35-40% 1-propanol and were spotted onto TLC plates (Si-60) and run in the solvent system C/M/HAc/W 25:15:4:2. The origin was scraped, GPIs eluted and partitioned between water and water-saturated butanol.

EXAMPLE 7

PURIFICATION OF GIPLS AND GPI PRECURSORS BY TLC

$2x10^3$[H]-glucosamine-labelled *P. falciparum* schizonts were extracted in CM (2:1) and CMW (1:1:0.3), Folch washed, partitioned between water and water-saturated butanol, and dried. Residues were separated by 2D TLC (1st dimension CMW 4:4:1, 2nd dimension Butanol/HAc/W 4:6:1), the plates scanned by Bertold Digital Autoradiograph scanner, and the structurally defined GPI peaks scraped and re-extracted. Phospholipids were resolved away from GPI peaks. Areas lying outside the identifiable GPI peaks were treated in the same way, as were sham plates. GIPLs of *L. mexicana* were purified to homogeneity. Briefly, promastigotes were extracted twice in CMW (1:2:0.8), the insoluble material removed by centrifugation, and the CMW phase partitioned with 0.6 volume water. The dried upper phase was chromatographed on Octyl-Sepharose as above, and eluted GIPLs further purified by HPTLC using CM/1N NH_4OH (10:10:3), and scrapings extracted with CMW (1:2:0.8). GIPL concentration and compositional purity was determined by GC-MS, following acid methanolysis and trimethylsilyl (TMS) derivatization. *myo*-Inositol content was measured following acid hydrolysis (6N HCl, 110°C, 16 h) and TMS derivatization, with selected ion monitoring for *m/z* 305 and 318. *scyllo*-Inositol was used as internal standard throughout.

EXAMPLE 8

GENERATION OF CHEMICAL AND ENZYMATIC HYDROLYSIS FRAGMENTS OF GPIS AND GIPLS

Purified, glucosamine-labelled *P falciparum* and *T. brucei* GPIs, in which all dpms were detected in the organic phase following butanol/water partitioning, were subject to base hydrolysis by suspension in methanol/ammonia 1:1 for 6 hours at 50°C, followed by partioning between water and water saturated butanol. Essentially 100% of label was then recovered from the aqueous phase. The aqueous phase was twice extracted with water-saturated butanol, lyophilized, and flash evaporated with methanol.

EXAMPLE 9

DEAE ANION EXCHANGE CHROMATOGRAPHY

GPIs were loaded onto a A DEAE column in 99% methanol, 1% water and washed with ten column volumes of solvent. They were subsequently eluted in 100mM Ammonium Acetate in 99% methanol, 1% water and dried under Nitrogen.

EXAMPLE 10

BIOGEL P4 SIZE-EXCLUSION CHROMATOGRAPHY

Base-hydrolysed GPI glycans were spiked with phenol red and blue dextran in 100mM Ammonium Acetate and further size-fractionated by passage through a 1cm x 1.2 metre Biogel P4 column equilibrated in 100mM Ammonium acetate in water. The column had previously been exhaustively calibrated by repeated analytical runs with GPI mixed with acid hydrolysed dextran markers to yield the relative elution position of glucose units detected by staining with orcinol in concentrated sulfuric acid. The column runs proved to be highly reproducible. For preparative purposes the dextran markers were omitted. The GPI peak was detected by scintillation counting of aliquots. Glycan concentration and compositional purity was determined by GC-MS, following acid methanolysis and trimethylsilyl (TMS) derivatization. *myo*-Inositol content was measured following acid hydrolysis (6N HCl, 110°C, 16 h) and TMS derivatization, with selected ion monitoring for *m/z* 305 and 318. *scyllo*-Inositol was used as internal standard throughout.

EXAMPLE 11

COUPLING OF GPI GLYCAN TO MALEIMIDE-ACTIVATED PROTEINS

GPIs were coupled to proteins by two methods. (i) GPIs were exposed to 1mM Traut's reagent (2-iminothiolane) in 40% 1-propanol, 60mM triethanolamine, 7mM potassium phosphate, 100mM NaCl, 1mM EDTA, pH 8.0 in the cold for 90 minutes under nitrogen. The sample was then desalted by gel filtration at 4°C through a small immobilized dextran

desalting column equilibrated in 40% 1-propanol in water. The propanol was dried off under nitrogen and the sample added to maleimide-activated KLH (Pierce) in coupling buffer (7mM potassium phosphate, 100mM NaCl, 1mM EDTA, pH 7.2) overnight followed by quenching with cysteine. (ii) (i) GPIs were exposed to 1mM Traut's reagent (2-iminothiolane) in 60mM triethanolamine, 30mM n-otg, 7mM potassium phosphate, 100mM NaCl, 1mM EDTA, pH 8.0 in the cold for 90 minutes under nitrogen. The sample was then desalted by gel filtration at 4°C through a small Biogel P4 column equilibrated in 30mM n-otg, 7mM potassium phosphate, 100mM NaCl, 1mM EDTA, pH 7.2 and added to maleimide-activated KLH (Pierce) in coupling buffer (7mM potassium phosphate, 100mM NaCl, 1mM EDTA, pH 7.2) overnight. The degree of conjugation was estimated by both by comparison of cpms before and after dialysis of the sample against PBS, or by use of Ellmans reagent to quantify sulfhydryl groups. Excess reactive groups were quenched with cysteine.

EXAMPLE 12
FACS ANALYSIS

$2x10^5$ cells in ice cold murine tonicity RPMI 1640 with 0.05% Sodium azide and 1% BSA were incubated with optimally titrated FITC- , biotin- or phycoerythrin-conjugated monoclonal antibodies to murine CD3, CD4, CD5, CD8, CD25, CD28, CTLA-4, CD44, CD69, Thy-1 (NIMR-1 or G7), and NK1.1 as required. After washing in the same medium the cells were counter-stained with 0.5μg/ml propidium iodide and analysed by FACSscan. Cells were sorted on FACStar II.

EXAMPLE 13
CYTOKINE CAPTURE ELISA ASSAYS

Cytokine levels were determined by specific capture ELISAs (Pharmingen). Treated and control samples were incubated at 37°C for 2 h in 96-well plates precoated with monoclonal anti-mouse IFN-γ, IL-2 and IL-4, followed by washing and probing under the

same experimental conditions with biotinylated second antibody anti-mouse IFN-γ, IL-2 and IL-4, followed by streptavidin-peroxidase. After addition of the substrate and color development, the plates were read by Titertek Multiscan MCC/340 automated ELISA reader. Cytokine mass was calculated by interpolating the results with the standard curve plotted with titrated recombinant mouse IFN-γ, IL-2 and IL-4 of known mass.

EXAMPLE 14

ELISA ASSAY

Antigen (tetanus toxoid, *P. falciparum* rCS, *P. berghei* rCS, $NANP_{40}$-BSA, FLU-BSA or BSA alone) at 10ug/ml in phosphate binding buffer was incubated overnight in 50ul volumes in flat-bottomed Immunlon 96-well plates, followed by extensive washing with buffer. The plates were blocked with 1% BSA in PBS for several hours. From a 1/32 dilution, sera were titrated two-fold in 1% BSA in PBS, and 50ul aliquots incubated in triplicate for 2 hours at room temperature, followed by extensive washing with 1% BSA, 0.05% Tween-20 in PBS. An aliquot of affinity purified, biotin-labelled isotype specific goat anti-mouse second antibody was incubated as above, followed by further washing and the addition of streptavidin-alkaline phosphatase. After 30 minutes the plates were washed again and colourimetric development initiated by the addition of p-Nitrophenylphosphate in diethanolamine buffer. Background binding to BSA-coated plates was determined in parallel. The titres derived are the last point giving values statistically different by two-way analysis of variance from non-specific binding by the same serum to the BSA-coated plates.

EXAMPLE 15

ANTIBODY FORMATION *IN VITRO*

Donor Balb/c *nu/nu* mice were primed twice with *P. berghei* SPZs or twice with LPS^{FLU}. 10^5 splenocytes were placed in culture for 7 days in the presence of IL-2, with and without antigen, anti-Class I, anti-Class II and anti-CD1 as indicated, with 10^4 $CD4^+$, $NK1.1^+$ cells positively selected by FACS sorting and Dynal Detachabead from spleens

of C57Bl6 donors primed to GPI-KLH. *P. berghei* SPZs or GPI-OVA^{FLU} were added with or without antibodies to CD1, MHC Class I or MHC Class II at various concentrations and cultured in RPMI 1640 + 10% FCS, $5x10^{-5}$ M β-ME and left for 8 days. Antigen-specific IgG production in the culture supernatant was determined by ELISA against recCS and fluoresceinated Dog serum albumin (Sigma) as capture antigen.

EXAMPLE 16

MHC II RESTRICTION IN THE ANTIBODY RESPONSE

This study determined that the antibody response to the native CS protein of malaria sporozoites is not Class II-restricted. Allogeneic bone-marrow irradiation chimeras, demonstrably unable to respond to the protein antigens Tetanus toxoid or recCS, were essentially equal to syngeneic chimeric controls in responding to irradiated sporozoites with the production of anti-CS IgG1 and IgG2 (Table 1). Nude mice cannot respond to sporozoites with anti-CS IgG, and as reported previously (3), passive transfer of cytolytic anti-CD4, but not anti-CD8, antibodies into euthymic animals abrogated anti-CS antibody formation in response to sporozoites. However, nude mice engrafted with irradiated neonatal allogeneic thymi produced anti-CS IgG1 and IgG2 in response to sporozoites at levels similar to recipients of syngeneic thymic implants, but did not respond to recCS or other protein antigens (Table 1). Class II$^{o/o}$ mice, lacking both Class II and Class II-restricted CD4$^+$ T cells, and reportedly incapable of mounting IgG responses to T-dependent protein antigens (4-6), responded to native but not recombinant CS protein with high titres of IgG1, IgG2a and IgG2b, similar to wildtype controls (Table 2). Together, these data demonstrate that thymically-derived CD4$^+$ cells are required for IgG formation in response to the native CS protein, but that the Class II MHC is not a restriction element in this response.

EXAMPLE 17

REQUIREMENT FOR CPI LIPID DOMAIN

To determine whether the GPI anchor could account for the difference in immunological behaviour of the native and recombinant proteins, GPIs purified from *Plasmodium falciparum, Leishmania mexicana* and *Trypanosoma brucei* were covalently linked in a molar ratio of 1:1 with haptenated Ovalbumin (GPI-OVA^{FLU}). In contrast to sham-OVA^{FLU} alone, GPI-OVA^{FLU} was able to induce anti-FLU and anti-OVA IgG1, IgG2a and IgG2b formation in MHC Class II$^{o/o}$ mice (Table 2). IgG responses in Class II mice were obtained only in response to OVA^{FLU} coupled to intact GPI, and not to OVA^{FLU} coupled to the deacylated GPI glycan, indicating the GPI lipid domain is required, and the glycan not sufficient, for the phenomenon (Table 2). However, GPI-OVA^{FLU} was unable to induce anti-FLU IgG responses in nude mice or euthymic animals treated with lytic anti-CD4 indicating that the GPI does not provide a sufficient signal to drive immunoglobulin class switch in the absence of CD4$^+$ cells. Isotype distributions similar to those obtained by immunization of Class II$^{o/o}$ mice with GPI-OVA^{FLU} were observed following exposure to the intact membrane form Variant Surface Glycoprotein of *T. brucei*, but not the deacylated soluble VSG derived by phospholipase C hydrolysis, confirming the requirement for GPI lipid domain and demonstrating that the phenomenon is generalizable to other GPI anchored proteins.

EXAMPLE 18

PROLIFERATIVE AND FUNCTIONAL RESPONSES TO GPI ANCHORED PROTEINS

The *in vitro* proliferative and functional responses to purified GPIs of splenocytes from wild-type and Class II$^{o/o}$ animals primed with either neo-GPI-anchored proteins, *Plasmodium falciparum* or *P. berghei* sporozoites, and blood stage *P. chabaudi* infection were examined. In all cases, compositionally pure GPIs (by GC/MS) elicited proliferative responses from splenocytes. The blastoid cells responding to GPI were predominantly

NK1.1$^+$, CD4^{int}, TCRα/β^{int}, Vα14+ and CD69+, and the FACS profiles revealed both a relative and absolute increase in this cell population. This was also accompanied by the production of high levels of IL-4. This unusual population of T cells was considerably expanded following exposure of mice to sporozoites. The proliferative and cytokine response of NK1+ CD4+ T cells to purified GPIs could be blocked by anti-CD1 monoclonal antibody 1B1(7).

EXAMPLE 19
ANTIBODY PRODUCTION

When splenocytes from sporozoite primed animals were exposed to sporozoites, they produced measurable levels of anti-CS IgG in the culture supernatant after 8 days. This antibody formation *in vitro* could be substantially blocked by anti-CD1 mAb 1B1, but less so by antibodies to Class II and not at all by anti-Class I. Furthermore, the NK1.1$^+$ cells were able to cooperate with B cells in CD1-restricted antibody formation to GPI-OVA^{FLU} but not OVA^{FLU}.

Wild-type and CD1$^{o/o}$ mice were exposed to sporozoites. Approximately 8-16 fold difference in anti-CS end-titres were detected, indicating that antibody formation to the native protein was reduced by approximately 90% in the latter animals, but in response to either TT or recCS was identical to wild-type controls (Table 3).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more said steps or features.

Table 1

MHC Class II is not a restriction element in IgG formation to SPZs.

Antigen:	TT	recCS	nativeCS
Test group			
BM chimeras			
Balb/c-----> Balb/c			
Animal#101	32768	32768	ND
Animal#103	65536	4096	ND
Animal#114	32768	8192	ND
Animal#107	131072	32768	ND
Animal#106	16384	ND	65536
Animal#111	32768	ND	32768
Animal#105	32768	ND	16384
Animal#121	8192	ND	32768
Animal#119	65536	ND	8192
Balb/c-----> Balb/K			
Animal#202	512	64	ND
Animal#203	128	128	ND
Animal#205	1024	256	ND
Animal#207	128	256	ND
Animal#208	256	ND	32768
Animal#211	512	ND	16384
Animal#212	256	ND	65532
Animal#217	1024	ND	65532
Thymic chimeras			
Balb/c-----> Balb/c			
Animal#1006	65536	6384	ND
Animal#1007	16384	16384	ND
Animal#1014	131072	4096	ND
Animal#1016	16384	ND	16384
Animal#1022	32768	ND	8192
Animal#1025	131072	ND	16384
Animal#1028	8192	ND	8192
Balb/c-----> Balb/K			
Animal#2004	32	64	ND
Animal#2005	16	32	ND
Animal#2006	< 16	< 16	ND
Animal#2010	128	< 16	ND
Animal#2014	32	ND	4096
Animal#2019	32	ND	16384
Animal#2020	16	ND	8192
Animal#2022	< 16	ND	8192

Table 2.

IgG responses to the recCS, native CS, OVA^{FLU} and $GPI\text{-}OVA^{FLU}$ in C57 wild-type, Class $II^{-/-}$ and $\beta_2M^{-/-}$ mice.

	Reciprocal titre to:			
	CS		FLU	
Primed with:	SPZs	recCS	OVA^{FLU}	$GPI\text{-}OVA^{FLU}$
1) Wild-type	4096	4096	4096	8192
	4096	8192	1024	512
	4096	8192	2048	512
	16384	16384		<16
2) Class $II^{-/-}$	512	<16	<16	128
	512	<16	<16	64
	2048	<16	<16	512
	2048	32	32	1024
3) $\beta_2M^{-/-}$	1024	8192	ND	ND
	2048	8192		
	2048	8192		
	4096	8192		

Table 3.

The IgG response to the native CS protein is reduced in CD1.$1^{0/0}$ mice

	Reciprocal anti-CS titre	
Immunized with:	SPZs	recCS
1) Balb/c	4096	4096
	4096	8192
	8192	8192
	16384	16384
2) Balb/c $CD1^{o/o}$	512	8192
	512	8192
	1024	8192
	2048	8192

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